Experimental Parasitology 135 (2013) 337–343

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Liposomal-lupane system as alternative chemotherapy against cutaneous leishmaniasis: Macrophage as target cell

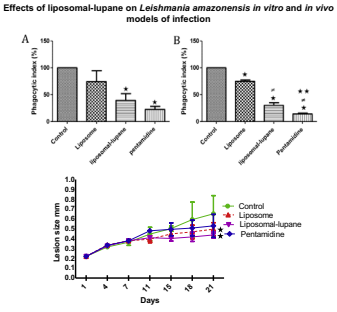
Neuza B. Barros [a,*], Vanessa Migliaccio [a], Valdir A. Facundo [b], Pietro Ciancaglini [c], Rodrigo G. Stábeli [a], Roberto Nicolete [a], Izaltina Silva-Jardim [a,d]

[a] Fundação Oswaldo Cruz (FIOCRUZ-RONDÔNIA), Porto Velho, RO, Brazil
[b] Departamento de Medicina, Universidade Federal de Rondônia, Porto Velho, RO, Brazil
[c] Departamento de Química, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto (FFCLRP), Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[d] Universidade Estadual de Santa Cruz, Departamento de Ciências Biológicas, Campus Soane Nazaré de Andrade, Ilhéus, BA, Brazil

HIGHLIGHTS

- Liposomal-lupane is able to decrease the growth/survival of Leishmania amastigotes.
- The formulation could inhibit the size of paw lesions in BALB/c-infected mice.
- Liposomes induce IL-12 and decrease IL-10 levels for the resolution of infection.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 February 2013
Received in revised form 12 July 2013
Accepted 19 July 2013
Available online 6 August 2013

Keywords:
Leishmania amazonensis
Lupane
Liposomes
Macrophages

ABSTRACT

Leishmania amazonensis causes human diseases that range from self-healing to diffusion cutaneous lesions. The chemotherapy of leishmaniasis requires long-term treatment and has been based on the use of pentavalent antimonials. Liposomes have been used as antileishmanial drug carries and have adjuvant activity in vaccines against several microorganisms, representing an important option to the development of new therapeutics for the disease. In this study, we developed a liposomal formulation containing lupane [3β,6β,16β-trihydroxylup-20(29)-ene], isolated from fruits of *Combretum leprosum* with pharmacological properties as antinociceptive, anti-inflammatory, antiulcerogenic and antileishmanial activities. The aim of the present study was to evaluate the efficacy of liposomal-lupane in *L. amazonensis*-infection model. Liposomes were prepared by the extrusion method with DPPC, DPPS and cholesterol at 5:1:4 weight ratio. The lupane (2 mg/mL) was added to the lipid mixture, solubilized in chloroform and dried under nitrogen flow. The activity of liposomal-lupane was conducted *in vitro* with mouse peritoneal infected macrophages. Furthermore, mice were infected in the right hind footpad with 10^5 stationary growth phase of *L. amazonensis* promastigotes. After 6 weeks, animals were treated with liposomal-lupane for 15 days by intraperitoneal injection. The evolution of disease was monitored weekly by measuring footpad thickness with a caliper. Three days after the treatment, peritoneal macrophages were collected, plated and production of the cytokines IL-10 and IL-12 was evaluated in supernatants of the cultures after 24 h. The results indicate that the liposomal system containing lupane achieved here is a promising tool to confer antileishmanial activity to infected macrophages.

1. Introduction

Leishmaniases are diseases caused by protozoa of the genus *Leishmania* and, clinically, may present as visceral, cutaneous or

* Corresponding author. Address: Laboratório de Biotecnologia Aplicada à Saúde, Fundação Oswaldo Cruz (FIOCRUZ-RONDÔNIA), Porto Velho, RO, Brazil. Fax: +55 693219 6009.
E-mail address: neuzabiguinati@gmail.com (N.B. Barros).

0014-4894/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exppara.2013.07.022

mucocutaneous forms (Bailey and Lockwood, 2007). It is an important zoonotic disease that occurs in the Old World and the Americas, characterizing itself as a serious public health problem in poor countries and a priority of the endemics of the World Health Organization (WHO). The American Cutaneous Leishmaniasis (ACL) can be present in three forms: cutaneous, mucocutaneous/cutaneous leishmaniasis and diffuse. Cutaneous leishmaniasis, the most common form, is characterized by skin lesions, ulcerated or not, however limited. The mucocutaneous leishmaniasis produces destructive lesions in the mucous membranes of the nose, mouth and pharynx. Typical lesions arise that involve the skin and mucous membranes and can occur metastasized to other parts of the body distant from the site of the bite. In Brazil, it is caused by *Leishmania (Viannia) braziliensis*, *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) guyanensis*. The diffuse cutaneous form is a rare and severe clinical form, which occurs in patients with deficient cellular immune response and is associated with infection with *L. amazonensis* (Piscopo and Mallia, 2006).

Over the past 90 years, the pentavalent antimony (Sb +5) is the drug of first choice recommended for the treatment of leishmaniasis (Rath et al., 2003). The high toxicity of this and other drugs, with reports of sudden death, is a limiting factor for their therapeutic use. Among the major adverse effects or side effects, the most common adverse reactions are pain, induration and sterile abscess at the site of application, as well as nausea, vomiting, dizziness, myalgia, headache, hypotension, hypoglycemia and hyperglycemia (Lima et al., 2007). Many compounds isolated from plants, such as chalcones, alkaloids, lignans, neolignans and triterpenes have been described as compounds with promising activity against protozoa (Dzubak et al., 2006). Regarding these phytocompounds, the triterpenesrepresent an expansion promising biologically active natural compounds whose potential is only partially exploited by the pharmaceutical industry (Dzubak et al., 2006). It has been reported its anti-inflammatory, analgesic, antimicrobial, antimycotic and immunomodulatory activities. The disadvantage of using triterpenoids, in fact, is the toxicity associated with its hemolytic and cytostatic properties (Dzubak et al., 2006). The triterpenes exhibit a wide structural diversity and can be classified into tetracyclic, pentacyclic and acyclic (Xu et al., 2004).

The lupane used in this study was isolated from *Combretum leprosum*, a kind honey scandent shrub with two to three feet tall, found in the states of Northeast and also in the states of Mato Grosso and Mato Grosso do Sul, Brazil (Facundo et al., 1993). The 3β,6β,16β-triidroxilup-20(29)-ene, a class of lupane triterpene, isolated from the flowers of *C. leprosum* showed antinociceptive activity and leishmanicidal activity against promastigotes forms of *L. amazonensis* (Teles et al., 2011). This lupane was able to reduce the infection rate and the amount of intracellular amastigotes in peritoneal macrophages.

Liposomes are microscopic spherical vesicles composed of phospholipids and natural or synthetic aggregate bilayer. They are comprised of one or more concentric lipid bilayers separated by aqueous layers and comprising an internal aqueous compartment. Since they are biodegradable, non-immunogenic and biocompatible, the structures are versatile, with features to reduce toxicity of substances. Also, they allow changes in their lipid composition, which address the liposome to specific cellular targets (Kalyankar et al., 2010). These drug carrier systems have been extensively studied and applied in the pharmaceutical, cosmetic, food, veterinary and clinical diagnosis. They represent a vesicular better therapy for certain diseases, such as for the treatment of leishmaniasis with liposomal amphotericin B, controlling the release of drug by increasing their pharmacokinetics and tissue reducing toxicity (Nicoletti et al., 2009).

The aim of the present study was to evaluate the efficacy of liposomal-lupane in both *in vitro* and *in vivo L. amazonensis*-infection models.

2. Materials and methods

2.1. Preparation of liposomes

Liposomes were prepared as described (Tempone et al., 2004) with modifications. The lipids used were dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS) and cholesterol (all reagents from Sigma–Aldrich) in the ratio 5:1:4. They were dissolved in a glass tube with 2 mL chloroform/methanol (Dinamica, São Paulo, Brazil) (1:1, v/v). Then, the tube was vortexed and subjected to ultrasonic bath repeatedly until the lipids dissolved completely. After the dissolution of lipids, a solution of chloroform/methanol was removed by evaporation, yielding a film of lipids on the inner wall of the tube. This film was vacuum dried in a desiccator for about 18 h and subsequently lipids were resuspended in 2 mL of 5 mM Tris–HCl, pH 7.5. The solution was then incubated for 1 h at 60° C with agitation every 10 min. The emulsion was subjected to an extruder (LiposoFastTM, Avestin, Ottawa, Canada) with a polycarbonate membrane with pores of 100–200 nm by 20 times to produce a population of liposomes of uniform size. For the preparation of liposomal-lupane, 2 mg of lupane triterpene were added to the lipids' solution, at the beginning of the preparation. The determination of the size of the liposomes was performed by dynamic light scattering using a Beckman Coulter Submicron Particle Analyzer Model (N5). The surface charge of the liposomes before and after permeation profile and the incorporation of lupane was evaluated from measurements of Zeta potential (ζ). This represents the residual negative charge on the particle surface. The samples were added to the cuvette containing the electrodes with a capacity of about 1 mL.

2.2. Animals

BALB/c mice, male, 8–10 weeks old, weighing 20–28 g, were obtained from the facility of the Instituto de Pesquisas em Patologias Tropicais (Ipepatro), Brazil. The animals were kept under standardized animal house conditions. The experiments were performed in accordance with standards established by the Ethics Committee on Animal Use upon approval of the research project under No. 2008/5.

2.3. In vitro infection of peritoneal macrophages

BALB/c mice were inoculated i.p. with 2 mL of 3% thioglycolate (Sigma–Aldrich) (8). After 4 days, the animals were euthanized by cervical dislocation and 5 ml of cold RPMI were injected into the peritoneal cavity and then recovered with a syringe. The peritoneal fluid was centrifuged at 250*g* for 10 min at 4 °C and the supernatant discarded. Then, the pellet was resuspended in 5 mL of RPMI medium and the cells obtained were counted in a Neubauer chamber. Macrophages (5×10^5/well) were plated in a 24 well plate containing sterile coverslips and incubated at 37 °C with humidified atmosphere containing 5% CO_2. Macrophages after 4 h incubation were washed twice with RPMI medium and then infected with promastigotes of *L. amazonensis* parasites (IFLA/BR/67/PH8), in the proportion of 5:1 macrophage. The infected cells were incubated "overnight" at 34 °C with a humidified atmosphere containing 5% CO_2. After this period, the wells were washed with RPMI and again treated with control liposomes and liposomal lupane (6 μg/mL). Pentamidine was used as reference drug. Infected cells were used untreated as a positive control of infection. The infected cultures

were maintained and treated for 24, 48 and 72 h at 34 °C. After these periods, the culture supernatants were collected and frozen for later cytokines' quantification. The infection rate and the number of intracellular parasites were evaluated by counting 100 macrophages in optical microscope – an increase of 1000×. Slides were stained by May Grünwald-Giemsa (Giaimis et al., 1992). The phagocytic index (Ranzani-Paiva et al., 2008) was determined using the formula: % of infected macrophages × total of Leishmania amastigotes.

2.4. In vivo infection and treatment of BALB/c mice

BALB/c mice were inoculated in the right hind paw with 10^5 promastigotes of *L. amazonensis* in PBS. After 6 weeks the treatment was started and the mice were separated, according to the treatment received in four groups. During 15 consecutive days each group received i.p. 100 μL of control and liposomal lupane (6 mg/kg). Pentamidine (4 mg/kg) was used i.p. as reference drug to treat the infection. As indication for treatment by the Brazilian Ministry of Health, Pentamidine was administered in alternated days. The progression of the lesion was monitored two times per week by measuring the size of the lesions with a pachymeter. The evolution of the disease in infected animals and the control group was evaluated weekly after initiation of treatment, for a period of 3 weeks by comparison of lesion size paw infected with the measurement of the uninfected contralateral paw. The results were expressed as the mean difference of the size of contralateral infected and uninfected paws. After 5 days of the end of the treatment, the animals were euthanized and peritoneal macrophages were collected, according to Section 2.3. The macrophage cultures were incubated at 37° C for 24, 48, 72 and 96 h. After each period, the supernatants were collected and stored at −20 °C for later cytokines' quantification.

2.5. Ex vivo determination of cytokines' production

BALB/c mice were infected according previously. After 6 weeks of infection, each group was treated for 15 days. On the 20th day, the animals were euthanized by cervical dislocation and ice-cold PBS was injected into the peritoneal cavity of each mouse to collect the macrophages. Then, 1×10^5 cells were incubated at 37 °C in periods of 48 and 96 h. After each period, the supernatants were collected and stored at −20 °C for cytokine assay.

The cytokines IL-10 and IL-12 were quantitated in the supernatants of cultures of peritoneal macrophages collected from the assay previously described (Section 2.4). For each cytokine, measurements were made using the enzyme immunoassay capture ELISA. For this we used the DuoSet kit, R&D Systems and BD Pharmingen according to the manufacturer's instructions.

2.6. Histopathologic analysis of the paw lesions in mice infected with L. amazonensis

Mice paws were collected and fixed in 10% formalin for a period of 24 h. After this, the paws were stored in PBS formalin (10% v/v) until histological processing. Subsequently, they were decalcified in EDTA solution at 10% in PBS for 4 days at a temperature of 4 °C. The samples were then subjected to paraffin embedding process for sagittal section (5 μm). The sections were dehydrated and diaphanized using increasing concentrations of alcohols and xylols, stained with hematoxylin and eosin and covered with coverslips for further analysis in light microscopy at 100× magnification to assess the standard and intensity of tissue changes and cellular infiltration.

2.7. Statistical analysis

Data obtained represent the mean and standard deviation of samples from two or three independent assays. One-way ANOVA test (Tuckey post-test) was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA) and analysis of variance to determine the level of significance set at $p < 0.05$.

3. Results

3.1. Characterization of the liposomes

The homogeneous generation and also the uniformity of liposomes formulations during the extrusion method were assessed using a particle size analyzer. Analyses by dynamic light scattering showed homogeneous population of both control and lupane liposomes. Table 1 shows sizes around 214.4 ± 0.12 nm and 146.3 ± 0.27 nm for control and liposomal-lupane, respectively. According to the data obtained, no significant difference in Zeta potential between control (−52.5 mV) and lupane (−51.1 mV) liposomes was achieved. The feasibility of liposomes containing lupane was determined by MTT assay using peritoneal macrophages from BALB/c mice after 24, 48 and 72 h incubation. Macrophages treated with liposomal lupane showed about 60% viability at high concentrations (above 100 μg/mL) (data not shown).

3.2. Liposomal-lupane decreases the growth/survival of the parasite inside the macrophages

Fig. 1(A) and (B) shows that, after 48 and 72 h incubation of infected macrophages with liposomal-lupane, a significant reduction of parasite survival was achieved (55.5% and 61.7%, respectively) compared to the control group. Pentamidine, as expected, presented greater significant reduction of 80.1% compared to untreated control group. Also, after 72 h, amastigote-infected macrophages incubated with liposomal-lupane presented significant reduction of the intracellular parasite survival compared with the control liposomes.

When the percentage of infected macrophages was correlated with total of Leishmania amastigotes, the phagocytic index was achieved. Fig. 1(C) and (D) presents these results and they reproduce the same phenomenon described above.

3.3. Treatment with liposomal-lupane decreases paw lesions size in L. amazonensis-infected mice

In order to evaluate the leishmanicidal effect of liposomal-lupane in animal model of cutaneous leishmaniasis, BALB/c mice were infected with 10^5 promastigotes of *L. amazonensis*. After 6 weeks, animals were treated for two consecutive days with control liposomes, liposomal-lupane (6 mg/kg) and Pentamidine (4 mg/kg) by i.p. route. The groups were treated every other day in accordance with material and methods (Section 2.4).

Table 1
Determination of the size and zeta potential of the liposomes.

Formulations	Vesicle size (nm)	Zeta potential (mV)
Control liposome	214.4 ± 0.12	−52.5
Liposomal-lupane	143.3 ± 0.27	−51.1

Size (nm) and Zeta potential (ζ) of control liposomes and those containing lupane. The measurements were performed with samples diluted in the range of four times, using HEPES buffer as a dispersion medium, at 25 °C. Values were obtained from two different batches.

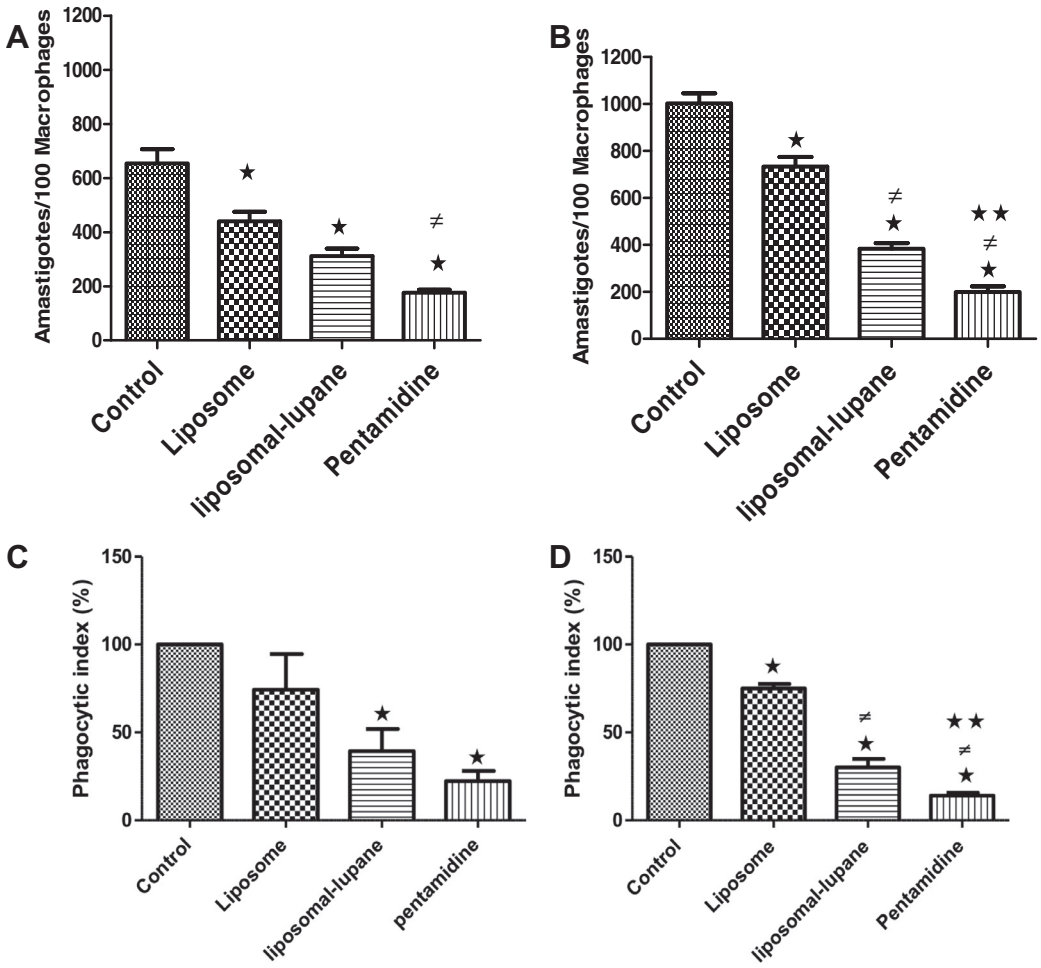


Fig. 1. Analysis of growth/survival of the parasite and phagocytic index in the macrophages after incubation with liposomes. Peritoneal macrophages were infected with promastigotes of *L. amazonensis* in stationary growth phase at a ratio of 5 parasites per cell. The peritoneal macrophages were *in vitro* infected and incubated 48 h (A) and 72 h (B) with 6 μg/mL of control liposomes or liposomal-lupane; Pentamidine was used as control. Phagocytic index of peritoneal macrophages after incubation with liposomes was achieved for 48 h (C) and 72 h (D). Each bar represents the mean ± S.D. of three independent experiments performed in triplicate. (*) ($p < 0.05$) Compared to control, (≠) ($p < 0.05$) compared to liposome and (**) ($p < 0.01$) compared to liposomal-lupane.

Fig. 2 shows the progression of the paw lesions, and also the profile of a lesion in mice without any treatment. It could be noted that on day 21, animals from control liposome, liposomal-lupane and Pentamidine groups showed a decrease in lesion size compared to the untreated control group. Mice that received liposomes containing lupane presented a statistically significant reduction in the lesion size by approximately 33% compared with untreated infected group.

3.4. Liposomal-lupane increases T_H1 protective cytokines ex vivo

Fig. 3 shows the results from cytokines' production by macrophages of infected and treated animals, as described (Section 2.4). Regarding IL-12 levels (Fig. 3(A)), *ex vivo* infected macrophages treated with both control liposome and liposomal-lupane showed a significant increase in the production of this cytokine when compared to the uninfected (control) and infected (untreated) macrophages. IL-10 levels (Fig. 3(B)) from infected macrophages treated with liposomal-lupane revealed a significant reduction in the levels of this cytokine compared to infected macrophages and also to those incubated with control liposomes only. As a reference drug, Pentamidine was able to decrease the levels of both cytokines by infected macrophages.

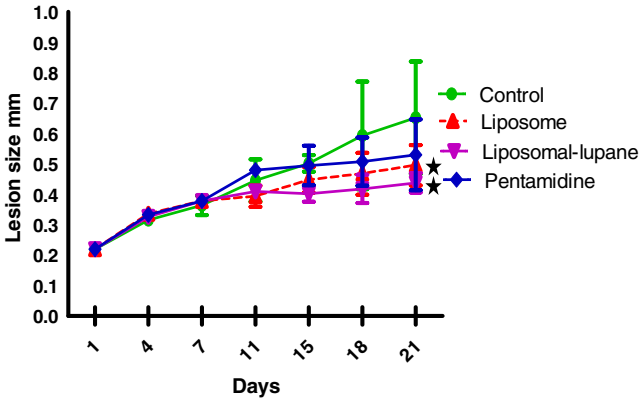


Fig. 2. Effect of the treatment with liposomal-lupane on *L. amazonensis* infected-mice. BALB/c mice were inoculated with promastigotes of *L. amazonensis* (1×10^5) and after 6 weeks were treated intraperitoneally for 15 days with 100 μL of liposome or liposomal-lupane (6 mg/kg) and Pentamidine (4 mg/kg). The size of the paw lesion was monitored with the aid of a slide caliper in two steps weekly over 21 days. The data represent the mean ± S.D. obtained for the lesions in two independent experiments, five animals per group. (*) Indicates a statistically significant difference from the 15th day of treatment comparing liposome and liposomal-lupane groups with control ($p < 0.05$).

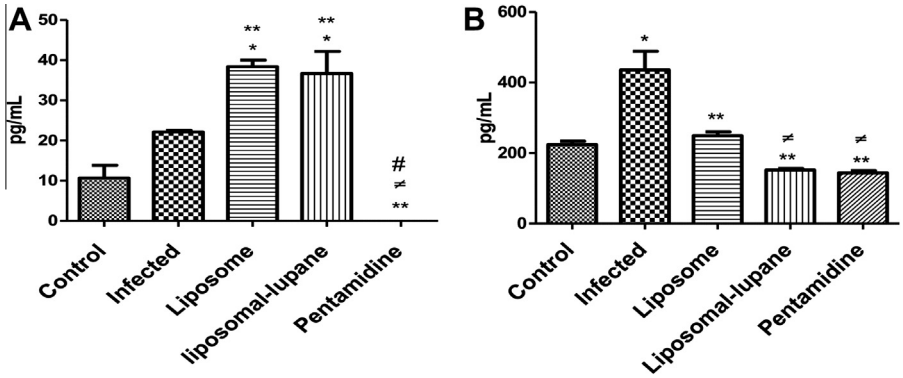


Fig. 3. *Ex vivo* production of the cytokines IL-12 and IL-10 in the supernatants of *L. amazonensis*-infected macrophages. Evaluation of *ex vivo* production of cytokines by peritoneal macrophages from BALB/c mice infected with *L. amazonensis* and treated with liposomes. (A) IL-12 after 48 h of incubation and (B) IL-10 production in the supernatants of macrophages after 96 h incubation. Cytokine concentrations were determined by ELISA. Each point represents the mean of cytokine production (pg/mL) ±S.D. (*) ($p < 0.05$) and (**) ($p < 0.01$) indicate a statistically significant difference comparing liposomes and liposomal-lupane with control infected group; (≠) ($p < 0.05$) liposomal-lupane compared with liposomes.

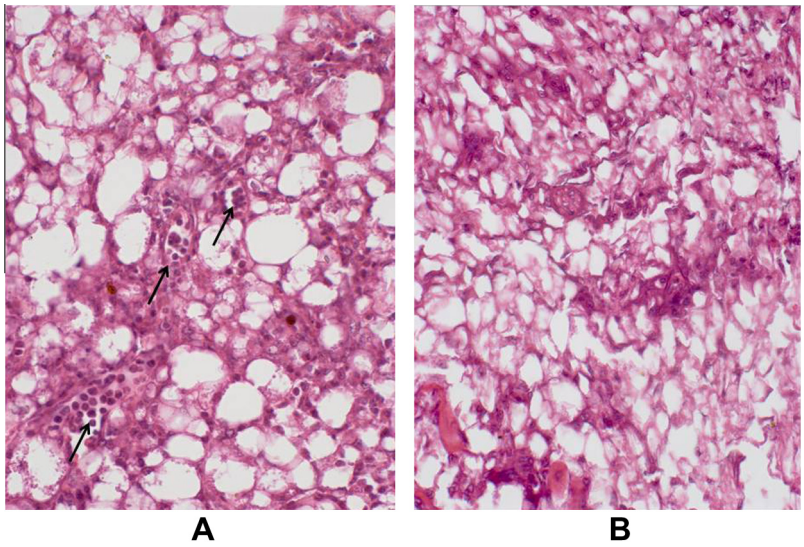


Fig. 4. Histopathologic analysis of the paw lesion in mice infected with *L. amazonensis*. (A) Control infected animals and (B) animals treated with liposomal-lupane. Arrows indicate amastigote nests. Magnifications 100×.

3.5. Liposomal-lupane decreases the number of amastigotes in infected macrophages

Histopathologic sections from Fig. 4 revealed that there are differences between the paw lesions from infected mice treated and not with liposomal-lupane. Numerous macrophage cytoplasms, large, clear and vacuolated were found in the dermis of the control group of mice infected with *L. amazonensis* (Fig. 4(A)). Macrophages were heavily infected, as indicated by the presence of a large number of amastigotes inside the vacuoles (arrows). In contrast, infected mice treated with the liposomal-lupane despite a vacuolization with destruction of the dermis showing no apparent parasites (Fig. 4(B)).

4. Discussion

Compounds encapsulated in liposomes become a viable alternative to diminish side effects unchained by classical drugs used to treat leishmaniasis, making their easier handling to be continued by the patient. The liposomal carrier vesicles have been important for the treatment of various diseases, including leishmaniasis, due to the fact that the encapsulated molecules can be retained intracellularly and directed to specific targets, reducing toxicity and preventing their degradation.

Regarding this drug delivery approach, liposomes have been used in experimental therapeutics as carriers for drugs (Kalyankar et al., 2010). Their layer composition has an important effect on its interaction with tissues and might be critical for the targeting to specific antigen presenting cells. Studies have demonstrated that liposomes containing DPPS enhances the production of IFN-γ, a T_H1-driving cytokine (Yotsumoto et al., 2007). Furthermore, DPPS liposomes entrapping pentavalent antimony have been shown to be captured by macrophages via scavenger receptors (Tempone et al., 2005). The tendency of liposomes to be captured by the mononuclear phagocytic system may be an advantage in treating a variety of intracellular infectious diseases. The increase of 200–700 times in the efficacy using liposomal pentavalent antimonials was observed in the treatment of visceral leishmaniasis in mice infected with *Leishmania donovani*. This fact can be attributed to the capture of liposomes by the organs and cells (tissue macrophages), which are located the parasites (Batista et al., 2007). Liposomes are the most drug delivery systems studied for leishmaniasis when compared to any other parasitic disease mainly due to the fact that

Leishmania colonizes the macrophages, target cells that contain amastigotes forms, and also responsible for the removal of the liposomes *in vivo*.

In the context of alternative antileishmanial therapies, many compounds isolated from plants, such as chalcones, alkaloids, lignans, neolignans and triterpenes have been described as compounds with promising activity against protozoa (Izumi et al., 2008). Regarding triterpenes, it has been reported its anti-inflammatory, analgesic, antimicrobial, antimycotic and immunomodulatory activities. The disadvantage of using triterpenoids, in fact, is the toxicity associated with its hemolytic and cytostatic properties (Dzubak et al., 2006).

Recently, it was showed that the viability of peritoneal macrophages from BALB/c mice decreased by 50% when they were incubated with the lupane solution (concentration at 10 μg/mL or above) (Teles et al., 2011). However, the effect of encapsulated lupane on macrophage activation has not yet been determined. In order to be considered ideal for therapeutic use, liposomes should provide molecule protection against metabolic processes and adjacent tissues, and also increase drug uptake by target cells (Kalyankar et al., 2010).

In this study, the size distribution of the liposomal vesicles constructed showed that both control and those liposomes containing lupane had a size greater than 100 nm, and also presented homogeneous populations of the vesicles formed. Determination of Zeta-potential can also be useful in elucidating the relationship of the charges of encapsulated drugs with the liposomal vesicles (Alonso et al., 1991). When analyzed, both formulations presented results around −52.0 mV. The Zeta-potential reflects the surface potential of particles, which is influenced by changes in the interface with the dispersing medium, due to decoupling of functional groups on the particle surface or adsorption of ionic species present in the aqueous dispersion (Mosqueira et al., 2000). The results showed that when the lupane was added to the vesicles to form liposomal structure, the negatively charged surface was maintained during the process and no further ion change was produced.

Teles et al. (2011) showed that the triterpene lupane, as a free non-encapsulated compound at a concentration of 5 mg/mL, presented a potent inhibitory activity on *L. amazonensis* promastigotes proliferation (IC50 = 3.3 mg/mL). Based on this toxic activity, for the first time we have proposed this study regarding the effect of lupane encapsulated into liposomes on the survival/growth of intracellular *L. amazonensis* amastigotes in order to correlate the antileishmanial activity elicited by lupane (free in solution or encapsulated) on both promastigote and amastigote forms. The liposomal-lupane reduced the amount of intracellular amastigotes and also the phagocytic index elicited by macrophages. These data therefore suggest that the uptaked liposomal-lupane affects the parasitic status of amastigotes inside macrophages, demonstrating also its leishmanicidal activity indirectly. Moreover, the results from the treatment of *L. amazonensis*-infected mice with liposomes showed that there was a decrease in the size of paw lesions (33%), monitored over a period of 21 days, when compared to infected and untreated groups. In this context of parasite infection inside the macrophages and paw lesions it can be pointed out the study with more than 30 cases of ACL in the Amazon region, where the main finding was the presence of large amounts of vacuolated macrophages, richly parasitized, also with lymphocytic cellular infiltrate (Silveira et al., 2005). The main histological characteristic of the infection by *L. amazonensis* is the accumulation observed in the dermis with numerous macrophages containing a large vacuole in the cytoplasm filled with amastigotes. With this regard, in this study, liposomes could release lupane inside the infected macrophages and provide clear decrease in the number of amastigote nests.

Taking together, our *in vitro* and *in vivo* results show that encapsulated lupane can act as an efficient antileishmanial therapeutic for controlling the parasite infection and also its intracellular permanence inside the macrophages.

The control of leishmaniasis in initial steps of the infection and during chemotherapy is dependent on macrophage activation mediated by T cells. The process of signaling between macrophages, lymphocytes and dendritic cells is accomplished primarily through the release of cytokines, which stimulate the production of multiple costimulatory molecules in the membrane surface and toxic reactive molecules against Leishmania amastigotes inside the macrophages. The activity of the parasite subversive process can take place by inhibiting production of IL-12, which is necessary for antileishmanial activity of macrophages (Kedzierski et al., 2009). The production of this cytokine by macrophages features a T_H1 response that is associated with resistance to infection, since this type of response is represented by cells producing IL-12, TNF-α and IFN-y cytokines (Murray et al., 2002).

The results from *ex vivo* experiments showed that the liposomes containing lupane were able to stimulate an increase in IL-12 production by *L. amazonensis*-infected macrophages and also, on the other hand, to reduce the production of IL-10. Both in *in vitro* (data not shown) and *ex vivo* experiments, it could be observed a decrease of this cytokine production by macrophages treated with liposomal-lupane. It has been well documented the role of IL-10 for contributing to disease progression in leishmaniasis, disabling T_H1 responses (Trinchieri et al., 2003; Saha et al., 2006) and acting simultaneously with IL-4 in the deactivation of infected macrophages, making them susceptible to the action of *Leishmania* (Murray et al., 2002).

In light of this context and other features in combination using liposomes to treat intracellular infections, our results can contribute to the advancement in the search for new therapeutic drugs and formulations, which may improve antimicrobial host defenses against the leishmaniasis, a disease that represents major public health problem, especially in underdeveloped countries.

Acknowledgments

This work was financially supported by Instituto de Pesquisas em Patologias Tropicais (IPEPATRO)/Fundação Oswaldo Cruz (Fiocruz Rondônia) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. We would like to thank Juliana Furtado, Sharon Rose Aragão Macedo, Sulamita Setúbal Silva, Leandro Moreira Dill Soares and Anderson Kayano for technical assistance in some experiments.

References

Alonso, M.J., Losa, C., Calvo, P., Vila-Jato, J.L., 1991. Approaches to improve the association of amikacin sulphate to poly(alkylcyanoarylate) nanoparticles. Int. J. Pharm. 68, 69–76.

Bailey, M.S., Lockwood, D.N., 2007. Cutaneous leishmaniasis. Clin. Dermatol. (Philadelphia) 25 (2), 203–211.

Batista, C.M., Carvalho, C.M.B., Magalhães, N.S.S., 2007. Lipossomas e suas aplicações terapeutas: Estado da Arte. Revista Brasileira de Ciências Farmacêuticas 43 (2), 167–179.

Dzubak, P., Hajduch, M., Vydra, D., 2006. Pharmacological activities of natural triterpenoids and their therapeutic implications. Nat. Prod. Rep. 23, 341–394.

Facundo, V.A., Andrade, C.H.S., Silveira, E.R., 1993. Triterpenes and flavonoids from C. leprosum. Phytochemistry 32, 411–415.

Giaimis, J., Lombard, Y., Makaya-Kumba, M., 1992. A new and simple method for studying the binding and ingestion steps in the phagocytosis of yeasts. J. Immunol. Methods 154 (2), 185–193.

Izumi, E., Morello, L.G., Ueda-Nakamura, T., 2008. Antileishmanial activity of parthenolide, a sesquiterpene lactone isolated from *Tanacetum parthenium*. Exp. Parasitol. 118, 324–330.

Kalyankar, T.M., Khan, M., Rangari, N.T., 2010. Novel possibilities of development and therapeutic applications of Liposomes. J. Pharm. Res. 3 (8), 1880–1884.

Kedzierski, L., Sakthianandeswaren, A., Curtis, J.M., 2009. Leishmaniasis: current treatment and prospects for new drugs and vaccines. Curr. Med. Chem. 16 (5), 599–614.

Lima, E.B., Motta, J.O.C., Porto, C., 2007. Tratamento da Leishmaniose Tegumentar Americana. An. Bras. Dermatol. 82 (2), 114–124.

Mosqueira, V.C.F., Legrand, P., Pinto-Alphandary, H., 2000. Poly(D,L-lactide) nanocapsules prepared by a solvent displacement process: influence of the composition on physicochemical and structural properties. J. Pharm. Sci. 89, 614–626.

Murray, H.W., Lu, C.M., Mauze, S., 2002. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Infect. Immun. 70 (11), 6284–6293.

Nicoletti, S., Seifert, K., Gilbert, I.H., 2009. N-(2-hydroxypropyl) methacrylamide–amphotericin B (HPMA-AmB) copolymer conjugates as antileishmanial agents. Int. J. Antimicrob. Agents 33 (5), 441–448.

Piscopo, T.V., Mallia, A.C., 2006. Leishmaniasis. Postgrad. Med. J. 82 (972), 649–657.

Ranzani-Paiva, M.J.T., Santos, A.A., Dias, D.C., 2008. Hematological and phagocytic response of fat snook, Centropomus parallelus, reared in net cages, before and after inoculation with *Saccharomyces cerevisiae*. Bioikos 22 (1), 29–35.

Rath, S., Trivelin, L.A., Imbrunitol, T.R., 2003. Antimoniais empregados no tratamento da leishmaniose. Química Nova 26 (4), 550–555.

Saha, S., Mondal, S., Banerjee, A., 2006. Immune responses in kala-azar. Indian J. Med. Res. 123 (3), 245–266.

Silveira, F.T., Lainson, R., Corbett, C.E.P., 2005. Further observations on clinical, histopathological, and immunological features of borderline disseminated cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis*. Mem. Inst. Oswaldo Cruz 100 (5), 525–534.

Teles, C.B.G., Moreira, L.S., Silva, A.A.E., 2011. Activity of the lupane isolated from *Combretum leprosum* against Leishmania amazonensis promastigotes. J. Braz. Chem. Soc. 22 (5), 936–942.

Tempone, A.G., Mortara, R.A., Andrade Jr., H.F., 2004. Therapeutic evaluation of free and liposome-loaded furazolidone in experimental visceral leishmaniasis. J. Antimicrob. Chemother. 60, 526–534.

Tempone, A.G., Perez, D., Rath, S., 2005. Targeting *Leishmania* (*L.*) *chagasi* amastigotes through macrophage scavenger receptors: the use of drugs entrapped in liposomes containing phosphatidylserine. J. Antimicrob. Chemother. 54, 60–68.

Trinchieri, G., Pflanz, S., Kastelein, R.A., 2003. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. Immunity 19 (5), 641–644.

Xu, R., Fazio, G.C., Matsuda, S.P.T., 2004. On the origins of triterpenoid skeletal diversity. Phytochemistry 65 (3), 261–291.

Yotsumoto, S., Kakiuchi, T., Aramali, Y., 2007. Enhancement of IFN-gamma production for Th1 cell therapy using negatively charged liposomes containing phosphatidylserine. Vaccine 25 (29), 5256–5262.